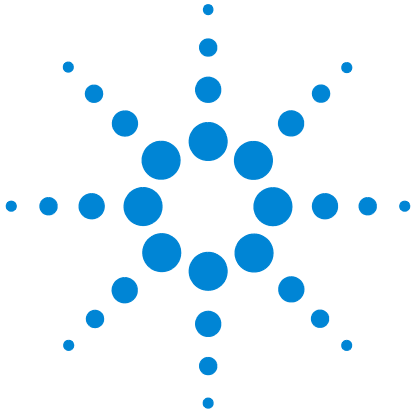

Agilent Seahorse XF Glycolysis Stress Test Kit

User Guide
Kit 103020-100

Agilent Technologies

Notices

Manual Part Number

103020-400

Kit Part Number

103020-100

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Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610 USA

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CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

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A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

Contents

1 Introduction

The Agilent Seahorse XF Glycolysis Stress Test is the standard assay for measuring glycolytic function in cells. By directly measuring the extracellular acidification rate, (ECAR), see Figure 1 on page 7. The Seahorse XF Glycolysis Stress Test provides a standard and comprehensive method to assess the key parameters of glycolytic flux: Glycolysis, Glycolytic Capacity, Glycolytic Reserve, as well as nonglycolytic acidification. (Refer to the "Glossary" on page 9 for more details.)

Introduction

Glycolysis and oxidative phosphorylation are the two major energy-producing pathways in the cell. Most cells possess the ability to switch between these two pathways, thereby adapting to changes in their environment. Glucose in the cell is converted to pyruvate (referred to as glycolysis), and then converted to lactate in the cytoplasm, or CO_2 and water in the mitochondria. The conversion of glucose to pyruvate, and subsequently lactate, results in a net production and extrusion of protons into the extracellular medium (Figure 2 on page 7). The extrusion of protons results in the acidification of the medium surrounding the cell.

The XF instrument directly measures the acidification rate, and reports this as ECAR. The assay workflow is as follows. First, cells are incubated in the glycolysis stress test medium without glucose or pyruvate and the ECAR is measured. The first injection is a saturating concentration of glucose. The cells utilize the glucose injection and catabolize it through the glycolytic pathway to pyruvate, producing ATP, NADH, water, and protons.

The extrusion of protons into the surrounding medium causes a rapid increase in ECAR. This glucose-induced response is reported as the rate of glycolysis under basal conditions. The second injection is oligomycin, an ATP synthase inhibitor. Oligomycin inhibits mitochondrial ATP production, and shifts the energy production to glycolysis, with the subsequent increase in ECAR revealing the cellular maximum glycolytic capacity.

The final injection is 2-deoxy-glucose (2-DG), a glucose analog, that inhibits glycolysis through competitive binding to glucose hexokinase, the first enzyme in the glycolytic pathway. The resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis. The difference between glycolytic capacity and glycolysis rate defines glycolytic reserve. ECAR, prior to glucose injection, is referred to as nonglycolytic acidification; caused by processes in the cell other than glycolysis.

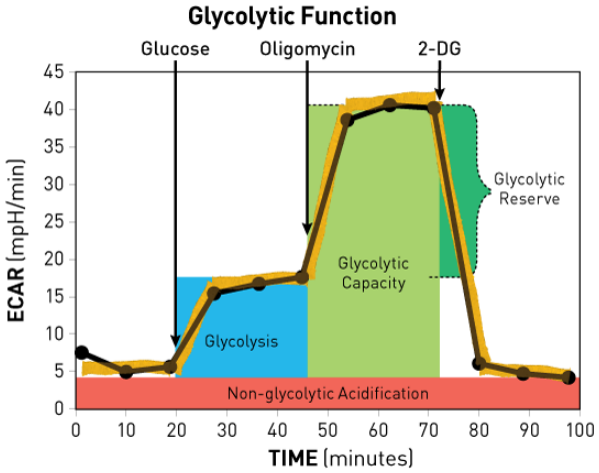

Figure 1 Agilent Seahorse XF Glycolysis Stress Test profile of the key parameters of glycolytic function. Sequential compound injections measure glycolysis, glycolytic capacity, and allow calculation of glycolytic reserve and nonglycolytic acidification.

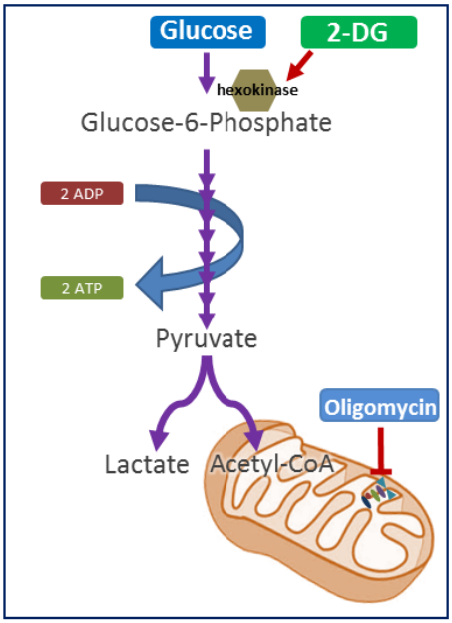

Figure 2 Agilent Seahorse XF Glycolysis Stress Test Modulators of Glycolysis. This diagram illustrates a simplified version of glycolysis and the sites of action of the kit components. Glucose fuels glycolysis. Oligomycin inhibits ATP synthase in the mitochondria resulting in an increased dependence on glycolysis. 2-DG is a competitive inhibitor of glucose, and functions to shut down glycolysis.

Table 1 Agilent Seahorse XF Glycolysis Stress Test Reagents (in order of injection).

Compound(s)	Target	Effect on ECAR
Glucose	Glycolysis	Increase
Oligomycin*	ATP Synthase Complex V	Increase
2-DG†	Glycolysis	Decrease

* Oligomycin is a mixture of Oligomycin A, B & C with Oligomycin A $\geq$ 60%.

† 2-DG may appear clear, opaque (white), or as a mix of white solid and clear liquid. Appearance does not affect performance.

Glossary

- **Glycolysis:** The process of converting glucose to pyruvate. The XF Glycolysis Stress Test presents the measure of glycolysis as the ECAR rate reached by a given cell after the addition of saturating amounts of glucose.
- **Glycolytic capacity:** This measurement is the maximum ECAR rate reached by a cell following the addition of oligomycin, effectively shutting down oxidative phosphorylation and driving the cell to use glycolysis to its maximum capacity.
- **Glycolytic reserve:** This measure indicates the capability of a cell to respond to an energetic demand as well as how close the glycolytic function is to the cell's theoretical maximum.
- **Nonglycolytic acidification:** This measures other sources of extracellular acidification that are not attributed to glycolysis.

2
Kit Information

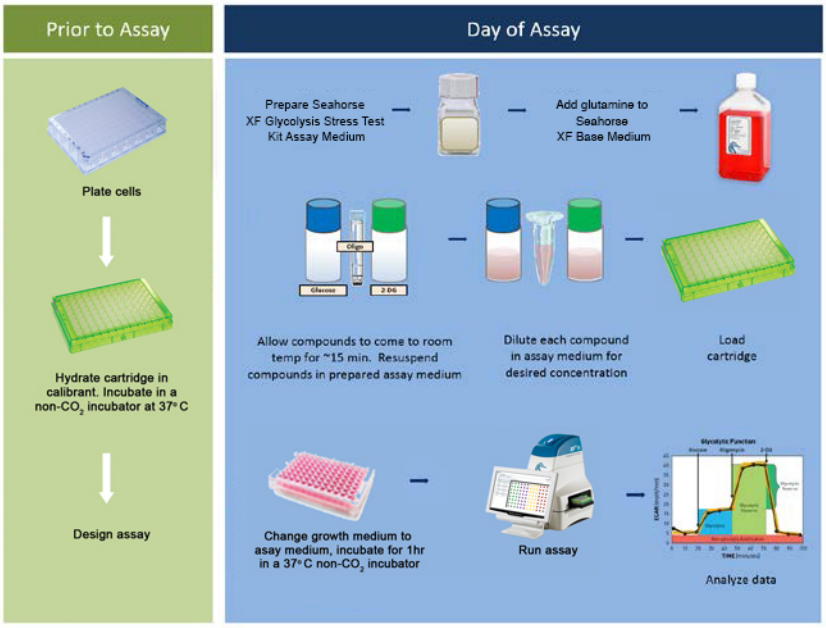

Figure 3 Agilent Seahorse XF Glycolysis Stress Test Assay Workflow.

Kit Contents

The Seahorse XF Glycolysis Stress Test Kit includes:

- Six foil pouches each containing oligomycin
- Six vials containing glucose
- Six vials containing 2-DG.

The kit reagents are sufficient for six complete Seahorse XF Glycolysis Stress Test assays.

Table 2 Agilent Seahorse XF Glycolysis Stress Test Kit contents.

Compound	Cap color	Quantity per tube
Glucose	Blue	300 µmol
Oligomycin	Light blue	72 nmol
2-DG	Green	1,500 µmol

Kit Storage

Product ships at ambient temperature, and should be stored at room temperature.

Table 3 Additional required items.

Agilent Seahorse XFe/XF96 or 24 Analyzer	Agilent Technologies	102745-100
Agilent Seahorse XF Base Medium	Agilent Technologies	102353-100-100 (2L), 103193-100 (100mL)
L- Glutamine	Sigma	G8540 or equivalent

Narrow p1000 pipette tips are recommended for reconstituting compounds within the tubes provided (for example, Fisherbrand™ SureOne™ Micropoint Pipet Tips, catalog #: 02-707-402)

3 Assay

Day Prior to Assay

1. Turn on the Seahorse XFe/XF Analyzer, and let it warm up to stabilize.
2. Plate cells at a previously determined density in the Seahorse XF Microplate using the appropriate cell culture growth medium. (Refer to Basic Procedure: Seeding Cells in Seahorse XF Culture Microplates available at www.agilent.com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfp-analyzer/basic-procedures-to-run-an-xfp-assay).
3. Hydrate a sensor cartridge in Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight. (Refer to Basic Procedure: Hydrating the Sensor Cartridge available at www.agilent.com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfp-analyzer/basic-procedures-to-run-an-xfp-assay).
4. Design experiment in Wave. Visit www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-xf-software.

Day of Assay

Prepare assay medium

1. Prepare the assay medium by supplementing Seahorse XF Base Medium. Agilent Seahorse recommends 1 mM glutamine, as a starting point; however, desired medium composition can be varied depending on cell type or *in vitro* culture conditions.
2. Warm the assay medium to 37 °C.
3. Adjust the pH to 7.4 with 0.1 N NaOH (Note: Agilent Seahorse recommends sterile filtration following pH adjustment).
4. Keep at 37 °C until ready to use.

Prepare stock compounds

NOTE Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.

1. The Seahorse XF Glycolysis Stress Test Kit includes:
   - Six foil pouches each containing oligomycin
   - Six vials containing glucose
   - Six vials containing 2-DG

   The kit reagents are sufficient for six complete XF Glycolysis Stress Test assays in a 96 or 24-well Seahorse XF Cell Culture Microplate.
2. Open a foil pouch containing oligomycin (light blue cap) and remove one vial containing glucose (blue cap) and one vial containing 2-DG (green cap) from the kit box.
3. Using a p1000 pipette, resuspend each component with prepared assay medium in volumes described in Table 4 on page 18. Gently pipette up and down (~10 times) to solubilize the compounds. Vortex the 2-DG to ensure that it goes into solution.

Figure 4 Removing reagent caps
Hold the tube in gloved hand and roll thumb in forward motion over the cap to loosen or, using the decapping tool provided, insert the tooth of decapper into the inner lip of the cap and gently rotate the tool backwards.

Table 4 Stock solutions.

Compound	Volume of assay medium	Resulting stock concentration
Glucose	3,000 µL	100 mM
Oligomycin	720 µL	100 µM
2-DG	3,000 µL	500 mM

Prepare compounds for loading in sensor cartridge

There are two approaches to loading the injection ports of the sensor cartridge:

- Constant loading volume/variable compound concentration
  This approach entails loading a constant volume of compound in each injection port and requires that each compound be prepared at a different concentration
- Constant compound concentration/variable loading volume
  This approach entails preparing the compounds at a constant concentration and requires that a different volume of each compound be loaded in the injection port

Table 5 and Table 6 on page 19 describes how to prepare to load the cartridges using both options. If using the constant volume option, media can be added directly to the glucose vial. If using the constant concentration option, no additional media is necessary. For oligomycin (with either loading option) pipette the stock volume into a conical tube and add the given volume of media. No media addition is necessary for 2-DG when running a standard assays.

Table 5 Compound preparation for loading sensor cartridge ports.

Agilent Seahorse XFe/XF96	Constant volume					Constant concentration				
	Starting well volume: 175 µL assay medium					Starting well volume: 180 µL assay medium				
Port A Glucose	(Final well) (mM)	Stock volume (µL)	Media volume (µL)	8X (Port) (mM)	Add to port (µL)	(Final well) (mM)	Stock volume (µL)	Media volume (µL)	10X (Port) (mM)	Add to port (µL)
	10	3,000	750	80	25	10	3,000	0	100	20
Port B Oligomycin	(Final well) (µM)	Stock volume (µL)	Media volume (µL)	9X (Port) (µM)	Add to port (µL)	(Final well) (µM)	Stock volume (µL)	Media volume (µL)	10X (Port) (µM)	Add to port (µL)
	1.0	270	2,730	9	25	1.0	300	2,700	10	22
Port C 2-DG	(Final well) mM	Stock volume (µL)	Media volume (µL)	10X (Port) (mM)	Add to port (µL)	(Final well) mM	Stock volume (µL)	Media volume (µL)	10X (Port) (mM)	Add to port (µL)
	50	3,000	0	500	25	50	3,000	0	500	25

Table 6 Compound preparation for loading sensor cartridge ports.

Agilent Seahorse XFe/XF24	Constant volume					Constant concentration				
	Starting well volume: 525 µL assay medium					Starting well volume: 500 µL assay medium				
Port A Glucose	(Final well) (mM)	Stock volume (µL)	Media volume (µL)	8X (Port) (mM)	Add to port (µL)	(Final well) (mM)	Stock volume (µL)	Media volume (µL)	10X (Port) (mM)	Add to port (µL)
	10	3,000	750	80	75	10	3,000	0	100	56
Port B Oligomycin	(Final well) (µM)	Stock volume (µL)	Media volume (µL)	9X (Port) (µM)	Add to port (µL)	(Final well) (µM)	Stock volume (µL)	Media volume (µL)	10X (Port) (µM)	Add to port (µL)
	1.0	270	2,730	9	75	1.0	300	2,700	10	62
Port C 2-DG	(Final well) mM	Stock volume (µL)	Media volume (µL)	10X (Port) (mM)	Add to port (µL)	(Final well) mM	Stock volume (µL)	Media volume (µL)	10X (Port) (mM)	Add to port (µL)
	50	3,000	0	500	75	50	3,000	0	500	69

Agilent Seahorse recommends 1 µM oligomycin; however, this can be varied if necessary given the specific sample conditions.

Load sensor cartridge

- **Standard Assay - no additional injection:** Load compounds into the appropriate ports of a hydrated sensor cartridge:

  Port A: Glucose

  Port B: Oligomycin

  Port C: 2-DG

- **Modified Assay – additional injection included:** To inject an additional compound prior to glucose, use port A for the desired compound and then load:

  Port B: Glucose

  Port C: Oligomycin

  Port D: 2-DG

Table 7 lists the appropriate volumes and concentrations for this injection scheme.

Table 7 Compound injection volumes involving an acute injection.

	Agilent Seahorse XFe/XF 96 Analyzer				Agilent Seahorse XFe/XF 24 Analyzer			
Port	Constant volume Starting well volume: 175 µL assay medium		Constant concentration Starting well volume: 180 µL assay medium		Constant volume Starting well volume: 525 µL assay medium		Constant concentration Starting well volume: 500 µL assay medium	
A	25 µL	8X	20 µL	10X	75 µL	8X	56 µL	10X
B	25 µL	9X	22 µL	10X	75 µL	9X	62 µL	10X
C	25 µL	10X	25 µL	10X	75 µL	10X	69 µL	10X
D	25 µL	11X	27 µL	10X	75 µL	11X	76 µL	10X

Prepare seahorse XF cell culture microplate for assay

1. Remove the cell culture microplate from the 37 °C CO_2 incubator and examine cells under a microscope to confirm confluence.
2. Remove the assay medium from water bath.
3. Using a multichannel pipette, change the cell culture growth medium in the cell culture microplate to warmed assay medium, and place the cell culture microplate into a 37 °C non-CO_2 incubator for 45 minutes to 1 hour prior to the assay.

Run the seahorse XF glycolysis stress test

Open the software and retrieve your saved assay template file. Follow the instructions below for your specific software.

If you are using XF software

1 Browse for, and open the saved design file then click **Run**.

2 Place the utility plate with the loaded sensor cartridge on the instrument tray. Calibration takes approximately 15-30 minutes.

NOTE Remove the cartridge lid and verify correct plate orientation

3 When prompted, replace the utility plate with the cell culture microplate then click **Start**.

NOTE Remove the microplate lid and verify correct plate orientation

If you are using wave

1 Browse and open the saved design file, select the **Review and Run** tab, then click **Start Run**.

2 When prompted, place the loaded sensor cartridge with the utility plate into the instrument, then click **I'm ready**. Calibration takes approximately 15-30 minutes.

NOTE Remove the cartridge lid and verify correct plate orientation

3 Following calibration, when prompted, click **I'm ready**. Load the cell culture microplate, and click **I'm ready** to run the assay.

NOTE Remove the microplate lid and verify correct plate orientation

Data Analysis

The Seahorse XF Glycolysis Stress Test Report Generator automatically calculates the Seahorse XF Glycolysis Stress Test parameters from the Wave data that has been exported to Excel. The Seahorse XF Stress Test Report Generator can be used with either a standard or modified stress test protocol, and provides a convenient, customizable, one-page assay summary.

The Seahorse XF Report Generator can be installed either alongside Wave or directly from the Seahorse Bioscience website. Visit www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-xf-report-generators to learn more about the Seahorse XF Stress Test Report Generators and download the User Guide.

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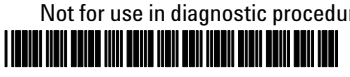

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